IMEKO International Conference on
Metrology for Archaeology and Cultural Heritage
Torino, Italy, October 19-21, 2016

Diet and Health in Middle Bronze Age Italy: a metaproteomic analysis of human dental calculus in two case-studies

Silvia Soncin[1], Jessica Hendy[2,3], Camilla Speller[3], Giorgio Manzi[1], Mary Anne Tafuri[1]

[1] *Dipartimento di Biologia Ambientale, Sapienza Università di Roma, Roma, Italy*
[2] *Department of Archaeology, Max Planck Institute for the Science of Human History, Jena, Germany*
[3] *BioArCh, Department of Archaeology, University of York, York, UK*

***Abstract* – Shotgun metaproteomics applied to dental calculus is a tool that is providing unprecedented insights in ancient diet and health reconstruction [1][2]. We apply a proteomic analysis of dental calculus deposits from individuals of two contemporaneous populations, Bovolone (Verona) and Sant'Abbondio (Pompeii), in order to provide more insight into the diet and health of individuals in middle Bronze Age Italy. Here we find that differences in protein preservation among individuals make comparing the two populations challenging. Nevertheless, we detect a number of dietary proteins, including wheats and eggs, which gains insight into food consumption practices during this period of social and economic change.**

I. INTRODUCTION

The Bronze Age has been long recognized as a period of profound changes in economic and social practices. At the basis of this social and cultural swing there were technological developments that resulted in agricultural and farming revolutions. However, population groups during this period often show different levels of complexity, and in Italy this variation has meant it has been difficult to define a relative chronology during the Early History. Italian territory is extremely complex and irregular, with different geographical barriers, which sometimes implied a lack of contact and cultural exchange among populations established in the Peninsula. With a few exceptions, each region adopted its own pottery, tools, weapons and funerary traditions, resulting in cultural traditions which, although connected temporally, were highly distinctive [3].

To further understand some of the dietary and health complexity of this period, we focus on two Italian Middle Bronze Age populations, located approximately 450 miles from one another, which appear to be culturally distinctive from one another (Fig.1). Archaeological, geographical, geological and anthropological data so far collected for the two sites are indicative of this distinction [4][5][6].

Bovolone, in the province of Verona, is a Bronze Age necropolis excavated a number of times since 1876. The individuals sampled for this study belong to the excavations carried out between 1981 and 1983 concerning the Middle Bronze Age area of the necropolis [4]. 64 graves were recovered during these campaigns, 29 of which are burials.

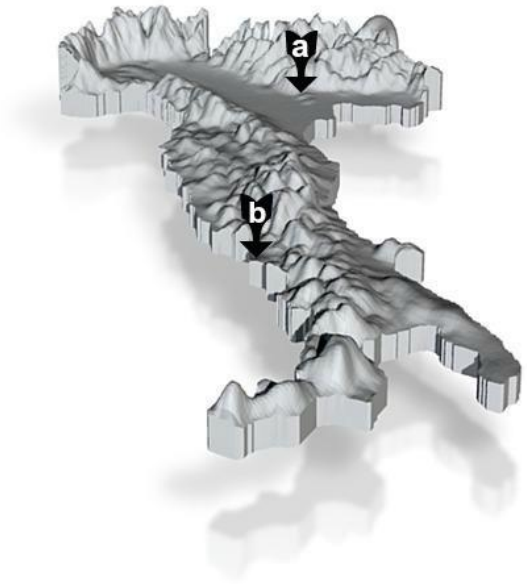

Fig 1. Location of Bovolone (a) and Sant'Abbondio (b).

The site is located in the alluvial cone of the Adige river, characterized by palaeo-rivers laid out in a wheel-and-spoke pattern, an ideal scenario for agriculture and animal husbandry. The assemblage of wild fauna, gathered from the adjacent settlement, excavated in 1996, is typical of a population in which hunting plays a secondary role while cattle herding prevails. The age-at-death study of cattle livestock at this site suggests a secondary products exploitation [5]. Tooth wear patterns are very common on individuals from Bovolone, and may

be characteristic of cereal consumption [4]. Osteological evidence for systemic disease, such as porotic hyperostosis and enamel defects, have a low incidence [4], which may indicate a relatively nutritious dietary regime.

Sant'Abbondio is a Middle Bronze Age necropolis situated in the modern city of Pompeii in the province of Naples. Excavations of Sant'Abbondio were led twice between 1992-1993, and 1996-1997 revealing 37 inhumations. The site is located on top of an isolated hill in the surrounding Pompeii plain just 1 km far from the sea, with the Sarno river running along the bottom of the hill and proximal to the Appennines. This makes Sant'Abbondio an ideal example of the prehistoric framework namely *Civiltà Appenninica*, based mainly on pastoral economy with seasonal semi-nomadism and on patriarchal structure described by Puglisi [7]. Similarly to Bovolone, the skeletal assemblage of Sant'Abbondio indicates a low incidence of metabolic disease, which may suggest the consumption of a balanced diet. A high incidence of caries and calculus was observed, which may be the result of a diet rich in carbohydrates, typical of agricultural populations [6]. Interestingly, pronounced differences in femural structure between male and female adults have been observed [6], which may support an idea of patriarchal structure across all Appennine territories based on pastoral activities [7].

Therefore, Bovolone and Sant'Abbondio populations present similar economical evolutions. Both of them seem to base their diet on cereal consumption, so demonstrating the adoption of these agricultural techniques. The management of livestock appears central in both the economies, despite with different practices: in Bovolone localised animal husbandry is more probable, while in Sant'Abbondio it seems to be related to seasonal transhumance. However, these sites also display different geographical and geological aspects, which might have played an important role in the diversification of harvest and disease outbreaks. In order to explore diet and disease in these populations, we explored the biomolecular analysis of ancient dental calculus.

Dental calculus (mineralized plaque) has often been studied in its own right as a dental pathology in the archaeological record [8]. However, recently ancient dental calculus has become recognised as one of the richest sources of ancient biomolecules, revealing insights into the composition of the oral microbiome and as a reservoir for dietary biomolecules [9][10][11]. Dental calculus is the mineralized form of dental plaque, a bacterial biofilm on the tooth surface containing commensal oral bacteria, pathogenic microorganisms, as well as host (human) biomolecules and dietary debris. Owing to its mineralised state, dental calculus is typically well preserved on archaeological skeletons. Ancient dental calculus has been extensively explored as a reservoir for microfossil remains [9][12], but recently with advances in ancient DNA and ancient protein analysis, is increasingly being explored to further understand the health and diets of past individuals. In this study we have explored a shotgun metaproteomics approach, which involves the extraction and analysis of the protein content of dental calculus. In the wake of burgeoning mass spectrometry technology, this approach is increasingly a valuable tool for identifying aspects of ancient health and diet on a direct, individual level [10] [11]. Here we apply this approach to dental calculus deposits from both Bovolone and Sant'Abbondio, in order to identify and compare any evidence of food consumption practices, as well as explore the oral and respiratory health of individuals in Middle Bronze Age Italy.

II. MATERIALS AND METHODS

We performed a shotgun metaproteomic analysis of 9 samples of dental calculus; 6 samples from Bovolone, and 3 from Sant'Abbondio. Deposits of dental calculus were removed the tooth surface using a sterile dental scaler. Only *supragingival* calculus has been sampled, predominately from premolars and molars (Fig. 2), and collected in 2.0 mL tubes. We used between 1.9 and 26.7 mg of dental calculus for protein extraction.

Fig. 2. Bovolone, burial 56 (T56). Calculus sampled from the labial surface of the upper incisors.

Samples of dental calculus were ground using sterile micropestles, and demineralized in EDTA following [10]. We then applied a GASP (Gel-Aided Sample Preparation) protocol, modified for ancient mineralized samples [13][14]. Extractions were performed in a dedicated clean room for ancient protein extraction at BioArCh, Department of Archaeology at the University of York. Extracted peptides were analysed using liquid chromatography tandem mass spectrometry (Q-Exactive)

at the Mass Spectrometry laboratory of the Target Discovery Institute at the University of Oxford. Spectra were converted in the Mascot generic format (MGF) using ProteoWizard version 3.0.10051, using 100 most intense peaks, and spectra searched using Mascot (Matrix Science™). Peptides were searched fully tryptically against UniProt, with a peptide tolerance set at 10 ppm with one missed cleavage and an MS/MS ion tolerance of ± 0.07 Da. Propionamide(C) was set as fixed peptide modification and Acetylation (Protein N-term), Deamidation (NQ), Oxidation (M), Propionamide (K), Propionamide (N-term) as variable modifications. Each Mascot search was filtered with ion score cut-off of 25 and a false discovery rate of 5%. Only proteins identified by Mascot with two or more peptides were considered. Peptides were searched using BLASTp to verify taxonomic assignments.

III. RESULTS AND DISCUSSION

Looking at the number of spectral queries produced for each sample, is evident that using more sample weight for protein extraction does not necessarily yield more peptide spectra (Fig.3). This variation in protein preservation means that making inter-population comparisons is challenging.

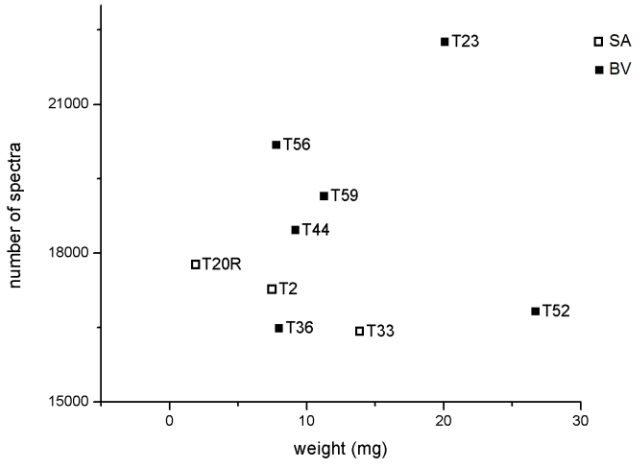

Fig.3. Scatter plot of number of spectra per mg of sample.

Pie charts (Fig.4) show the percentages of the different origin proteins, in particular pathogenic oral microbiome, non-pathogenic oral microbiome, host proteins and dietary proteins.

A. Pathogenic bacterial species

Proteins of pathogens were found in abundance in Bovolone while they are poorly represented in Sant'Abbondio. Immunoglobulin G-binding protein belonging to *Streptococcus* sp. *Group G* (GGS) was found in all the samples. GGS has been recognized as part of the normal human oral flora and associated with some infections like pharyngitis [15]. GGS is also the unique pathogen found in Sant'Abbondio. Among pathogens observed in Bovolone, are worth noting *Francisella tularensis*, which was found in all the samples and is the causative agent of tularemia, even in three of them the subspecies *nocivida* has been matched, which is rarely virulent [16]; *Haemophilus influenzaea*, a common resident of the nasopharyngeal mucosa from which enters the bloodstream and spread to the rest of the body causing a wide range of infections [17]; *Propionibacterium acnes*, a commensal of human skin and at the beginning associated only with acne vulgaris, has been found to be implicated in a large number of human pathologies such as endocarditis, hypostasis, pustulosis, pulmonary angitis, allergic alveolitis and others [18]; *Porphyromonas gingivalis*, which is an habitant of the oral cavity and the major causative agent of periodontal diseases [19]; *Corynebacterium diphtheriae* was detected matching different proteins. It is the causative agent of diphteria, a severe disease that interest the respiratory tract [20].

B. Non-pathogenic microbiome

Many non-pathogenic bacteria were detected in both the assemblage but most of them, being soil-dwelling, are not reported here. Three of them, from Bovolone samples, are not commonly found in soil and so shown below. *Bilophila wadsworthia* was found in three samples with Taurine-pyruvate aminotransferase protein. It is normally inhabitant of faeces and, occasionally, of saliva [21]. 60 kDa chaperonin of *Lactobacillus salivarius* was detected in one sample. This is a probiotic organism and a pathogens suppressor commonly found in mammalian digestive tract [22]. *Peptococcus niger*, detected with one protein in one sample, is part of the normal human umbilical and intestinal flora [23]. However, it should be stressed that many microbial species, especially non-pathogenic and non-commercial species, have no sequence information and are not represented in the database.

C. Host proteins

Human proteins make a large percentage of the total number of identified proteins in dental calculus from both sites. As has previously been detected in ancient dental calculus [1], many are involved in antimicrobial, responses, such as myeloperoxidase, peptidoglycan recognition protein 1, resistin, S100-A8 and A9, azurocidin, neutrophil elastase, cathepsin G, myelobastin and immunoglobulins. Many of these proteins are the result of the host response to a bacterial biofilm. For example, myeloperoxidase, occurring numerously (e.g. 94 peptide matches in T44) in all the Bovolone samples and in one individual from Sant'Abbondio is a major constituent of the azurophilic cytoplasmic granules of

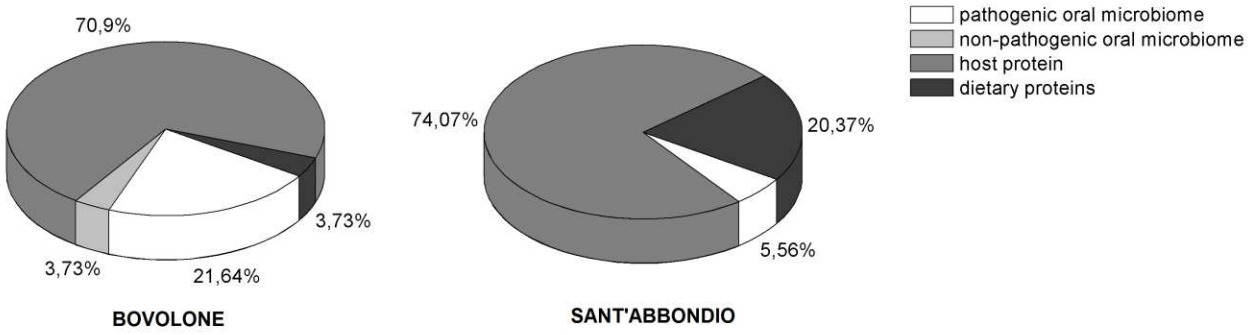

Fig.4. Pie charts showing the percentages of the different origin of proteins.

neutrophils and plays a central role to their microbiocidal activity [24]. Similarly, azurocidin is one of the proteins of azurophil granules implicated in antimicrobial activity, and is also a multifunctional inflammatory mediator [25]. Immunoglobulin γ-1 chain C region and Immunoglobulin κ chain C region are antibodies, central proteins of the humoral immunity [26]. Among proteins with anti-inflammatory activities we found α1-antichymotrypsin, a glycoprotein that inhibits the activity of proteases protecting tissues [27]; leukocyte elastase inhibitor, which inhibits polymorphonuclear neutrophils, in particular neutrophil elastase, PR3 and cathepsin G all found in neutrophil granules [28]; α1-antitrypsin, a protease inhibitor which protects tissues from enzymes of inflammatory cells [29]; α-2-macroglobulin, a large plasma protein that acts as an antiprotease [30]; and antithrombin III, which is a glycoprotein found in plasma that inactivates thrombin protease enzyme [31].Proteins with structural and other functions were detected. Many of these are involved in the matrix composition of host cells, and may be present as part of the plaque biofilm. For example, matrix extracellular phosphoglycoprotein is a protein of extracellular matrix of bone and dentin and regulates bone mineralization [32]; collagen α-2(I) chain, which consists in one of the chain of the fibrillary collagen found in connective tissues [33]; serum albumin, the most abundant in human blood plasma with transport functions [34]; and filaggrin is essential for the regulation of epidermal homeostasis and it is found in epithelial cells [35].

D. Dietary proteins

Among all the Bovolone proteins, two dietary proteins were detected. These are ovotransferrin and ovalbumin-related protein X with peptides matching uniquely to *Gallus gallus* (chicken). Both these proteins are found in egg whites [36]. However, it should be noted that egg proteins are often used in molecular laboratories as standards or molecular weight markers, so their interpretation should be considered with caution.

In contrast, more dietary proteins were identified in individuals from the Sant'Abbondio assemblage. Ovotransferrin and ovalbumin-related protein X from *Gallus gallus* were detected again, with the addition of avidin and vitelline membrane outer layer protein 1. Avidin is a vitamin B7 carrier produced in the oviducts and deposited in the albumen of the eggs [37]. Vitelline membrane outer layer protein 1, found in the vitelline membrane which function is to keep separate yolk and albumen and to act as an anti-microbial barrier, has a structural function [38]. In addition, two proteins belonging to wheats were detected in one sample of Sant'Abbondio, alpha-amylase inhibitor 0.53 and glutenin, high molecular weight subunit 12, both found expressed in the endosperm of the plant [39]. All wheat peptides identified match to the level of Triticeae, with most matching to the subtribe of Triticinae, and one, in particular, matching uniquely to *T. aestivum*, but also *T. turgidum* subsp. *durum*. These results directly indicate the consumption of wheat by individuals buried at Sant'Abbondio.

IV. CHALLENGES

Our results reveal a high level of variation in the number of peptide spectra produced from dental calculus samples in this study. Unrelated to the weight of the sample selected for analysis, this may indicate that decay mechanisms act on an individual level, resulting in substantial intra-population variation. As a result, this variation within populations makes inter-population comparisons challenging. Secondly, it is important to consider that ancient biomolecular analyses are susceptible to contamination, from the burial and lab environment. Whilst ancient proteins may be more robust than ancient DNA [40], we are still unaware of many mechanisms of protein survival.

ACKNOWLEDGEMENTS

We thank Luciano Salzani, Gianni De Zuccato and Alessandro Canci for permission to study the material from Bovolone. We thank Pierfrancesco Talamo for granting permission to analyse the Sant'Abbondio material. Thanks to Oliver Craig for providing access to laboratories at the University of York.

REFERENCES

[1] C. Warinner, J. F. M. Rodrigues, R. Vyas, C. Trachsel, N. Shved, J. Grossmann, A. Radini, Y. Hancock, R. Y. Tito, S. Fiddyment, et al. Pathogens and host immunity in the ancient human oral cavity. Nature genetics, 46(4):336–344, 2014a.

[2] C. Warinner, J. Hendy, C. Speller, E. Cappellini, R. Fischer, C. Trachsel, J. Arneborg, N. Lynnerup, O. E. Craig, D. Swallow, et al. Direct evidence of milk consumption from ancient human dental calculus. Scientific reports, 4, 2014b.

[3] A. M. B. Sestieri. L'Italia nell'età del bronzo e del ferro: dalle palafitte a Romolo (2200-700 a.C.). Carocci, 2010.

[4] L. Salzani. La necropoli dell'età del bronzo di Bovolone. Memorie del Museo Civico di Storia Naturale di Verona, Sezione Scienze dell'uomo. Museo Civico di Storia Naturale di Verona, 2010.

[5] U. Hohenstein, M. Bertolini, G Petrucci, and L. Salzani. L'insediamento dell'età del bronzo medio-recente di bovolone: risultati preliminari dell'analisi dei resti faunistici e dei manufatti in materia dura animale. *Padusa*, 46(46):109–122, 2010.

[6] M. A. Tafuri. Tracing Mobility and Identity: Bioarchaeology and Bone Chemistry of the Bronze Age Sant'Abbondio Cemetery (Pompeii, Italy). Archaeopress, 2005.

[7] S. M. Puglisi. La civiltà appenninica. Origine delle comunità pastorali in Italia. Sansoni Firenze, 1959.

[8] A. R. Lieverse. Diet and the aetiology of dental calculus. International Journal of Osteoarchaeology, 9(4):219–232, 1999.

[9] K. Hardy, T. Blakeney, L. Copeland, J. Kirkham, R. Wrangham, and M. Collins. Starch granules, dental calculus and new perspectives on ancient diet. Journal of Archaeological Science, 36(2):248–255, 2009.

[10] C. Warinner, J. F. M. Rodrigues, R. Vyas, C. Trachsel, N. Shved, J. Grossmann, A. Radini, Y. Hancock, R. Y. Tito, S. Fiddyment, et al. Pathogens and host immunity in the ancient human oral cavity. Nature genetics, 46(4):336–344, 2014.

[11] C. Warinner, J. Hendy, C. Speller, E. Cappellini, R. Fischer, C. Trachsel, J. Arneborg, N. Lynnerup, O. E. Craig, D. Swallow, et al. Direct evidence of milk consumption from ancient human dental calculus. *Scientific reports*, 4, 2014.

[12] A. G Henry and D. R. Piperno. Using plant microfossils from dental calculus to recover human diet: a case study from tell al-raq¯a'i, syria. Journal of Archaeological Science, 35(7):1943–1950, 2008.

[13] Fischer, Roman, and Benedikt M. Kessler. "Gel-aided sample preparation (GASP)—A simplified method for gel-assisted proteomic sample generation from protein extracts and intact cells." *Proteomics* 15.7 (2015): 1224-1229.

[14] GASP (Gel-Aided Sample Preparation) Protein Extraction Protocol, based on Fischer & Kessler (2014) modified for ancient mineralized samples, BioArCh bench-top protocol, University of York, UK.

[15] Tan, James S., and Thomas M. File. "Streptococcus species (Group G and Group C Streptococci, Viridans Group, Nutritionally Variant Streptococci)."

[16] Larsson, Pär, et al. "The complete genome sequence of Francisella tularensis, the causative agent of tularemia." Nature genetics 37.2 (2005): 153-159.

[17] Vidya R Devarajan, MD, Wesley W Emmons, Francisco Talavera, PharmD, PhD, Charles V Sanders, MD, Eleftherios Mylonakis, MD, PhD, Burke A Cunha, MD, MACP. "Haemophilus Influenzae Infections" eMedicine from WebMD. January 16,2007.

[18] Brüggemann, Holger, et al. "The complete genome sequence of Propionibacterium acnes, a commensal of human skin." Science 305.5684 (2004): 671-673.

[19] Naito, Mariko, et al. "Determination of the genome sequence of Porphyromonas gingivalis strain ATCC 33277 and genomic comparison with strain W83 revealed extensive genome rearrangements in P. gingivalis." DNA research 15.4 (2008): 215-225.

[20] Cerdeño - Tárraga, A. M., et al. "The complete genome sequence and analysis of Corynebacterium diphtheriae NCTC13129." Nucleic Acids Research 31.22 (2003): 6516-6523.

[21] Finegold, S., et al. "Clinical importance of Bilophila wadsworthia." European Journal of Clinical Microbiology and Infectious Diseases 11.11 (1992): 1058-1063.

[22] Neville, B. A., and P. W. O'Toole. "Probiotic properties of Lactobacillus salivarius and closely related Lactobacillus species." Future Microbiology 5.5 (2010): 759-774.

[23] Murdoch, D. A. "Gram-positive anaerobic cocci." Clinical microbiology reviews 11.1 (1998): 81-120.

[24] Klebanoff, Seymour J. "Myeloperoxidase: friend and foe." Journal of leukocyte biology 77.5 (2005): 598-625.

[25] Watorek, Wiesaw. "Azurocidin—inactive serine proteinase homolog acting as a multifunctional inflammatory mediator." (2003).

[26] Janeway, Charles A., et al. Immunobiology: the immune system in health and disease. Vol. 1.

Current Biology, 1997.
[27] Kalsheker, Noor A. "α 1-Antichymotrypsin." The international journal of biochemistry & cell biology 28.9 (1996): 961-964.
[28] Remold-O'Donnell, Eileen, Jean Chin, and Monica Alberts. "Sequence and molecular characterization of human monocyte/neutrophil elastase inhibitor." Proceedings of the National Academy of Sciences 89.12 (1992): 5635-5639.
[29] Gettins, Peter GW. "Serpin structure, mechanism, and function." Chemical reviews 102.12 (2002): 4751-4804.
[30] Strickland, Dudley K., et al. "Sequence identity between the alpha 2-macroglobulin receptor and low density lipoprotein receptor-related protein suggests that this molecule is a multifunctional receptor." Journal of Biological Chemistry 265.29 (1990): 17401-17404.
[31] Björk, Ingemar, and Steven T. Olson. "Antithrombin." Chemistry and biology of serpins. Springer US, 1997. 17-33.
[32] Nampei, Akihide, et al. "Matrix extracellular phosphoglycoprotein (MEPE) is highly expressed in osteocytes in human bone." Journal of bone and mineral metabolism 22.3 (2004): 176-184.
[33] Retief, E., M. I. Parker, and A. E. Retief. "Regional chromosome mapping of human collagen genes alpha 2 (I) and alpha 1 (I)(COLIA2 and COLIA1)." Human genetics 69.4 (1985): 304-308.
[34] He, Xiao Min, and Daniel C. Carter. "Atomic structure and chemistry of human serum albumin." Nature 358.6383 (1992): 209-215.
[35] Markova, Nedialka G, et al. "Profilaggrin is a major epidermal calcium-binding protein." Molecular and cellular biology 13.1 (1993): 613-625.
[36] Mann, Karlheinz. "The chicken egg white proteome." Proteomics 7.19 (2007): 3558-3568.
[37] Durance, T. D., and N. S. Wong. "Kinetics of thermal inactivation of avidin." Food research international 25.2 (1992): 89-92.
[38] Back, Joan F., et al. "Proteins of the outer layer of the vitelline membrane of hen's eggs." Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology 705.1 (1982): 12-19.
[39] Skylas, D. J., et al. "Proteome approach to the characterisation of protein composition in the developing and mature wheat-grain endosperm." Journal of Cereal Science 32.2 (2000): 169-188.
[40] E. Cappellini, M. J. Collins, and M. T. P. Gilbert. Unlocking ancient protein palimpsests. Science, 343(6177):1320–1322, 2014.